# THE EFFECT OF TRYPSIN ON REGENERATION INHIBITORS IN TUBULARIA[1]

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Regeneration can occur at all levels along the stem of the marine hydroid, *Tubularia*. In cut pieces of stem the end that was nearest the hydranth usually regenerates a new hydranth, whereas the other extremity of the piece usually regenerates a new proximal end.

The region formed first during reconstitution is the hydranth. Once it is formed it can exert an influence on the other regions of the stem fragment. The hydranth is referred to as the dominant region because it controls the morphogenetic processes of the other regions in its field. (The term *field* implies the area in which some agent is at work in a co-ordinated way establishing an equilibrium within the area [Child, 1941].). In the intact animal the hydranth has the ability to prevent differentiation of another hydranth in the field it dominates. When the hydranth is removed from a stem fragment, regeneration occurs.

Rose and Rose (1941) found that there must be enough cut surface of the stem exposed to the sea water environment to allow escape of an inhibiting substance. They collected "inhibitor water" from aerated sea water containing many cut hydranths. This water was capable of inhibiting regeneration of newly cut stems. Heating destroyed the inhibitory effect of the water.

Fulton (1959) collected inhibitor water in low concentrations of either streptomycin or penicillin. Since this water did not affect regenerating stems, he suggested that the activity of inhibitor water might be due to bacteria or their metabolic products. Since Fulton's re-examination of the inhibitor, Tweedell (1958b) has produced a bacteria-free inhibitor water. Rose (1955, 1957, 1961, 1963) has been able to demonstrate inhibitory effects with grafts of living tissue when bacterial action was not a factor. Grafts of hydranth primordia, correctly oriented in a distal position, suppress the development of homologous portions of the host. Tweedell (1958a, 1962) and Powers (1961) have shown that tissue extracts from adult hydranths can inhibit reconstitution of stems. Finally, Rose (1963) has described an inhibiting substance obtained from primordia that can be moved electrophoretically. The present experiments were designed to study further this substance that can be extracted from regenerating *Tubularia* and can inhibit regeneration.

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## Methods

*Tubularia crocea* was used as an experimental animal because of its rapid rate of regeneration and its easy availability near the Marine Biological Laboratory at Woods Hole, Massachusetts, where this research was done.

Colonies of *Tubularia* were collected freshly for each experiment from the Cape Cod Canal. The colonies were kept cool during transport by immersing plastic bags filled with ice cubes in their container. The animals were brought into the laboratory as soon as possible and placed in running aerated sea water.

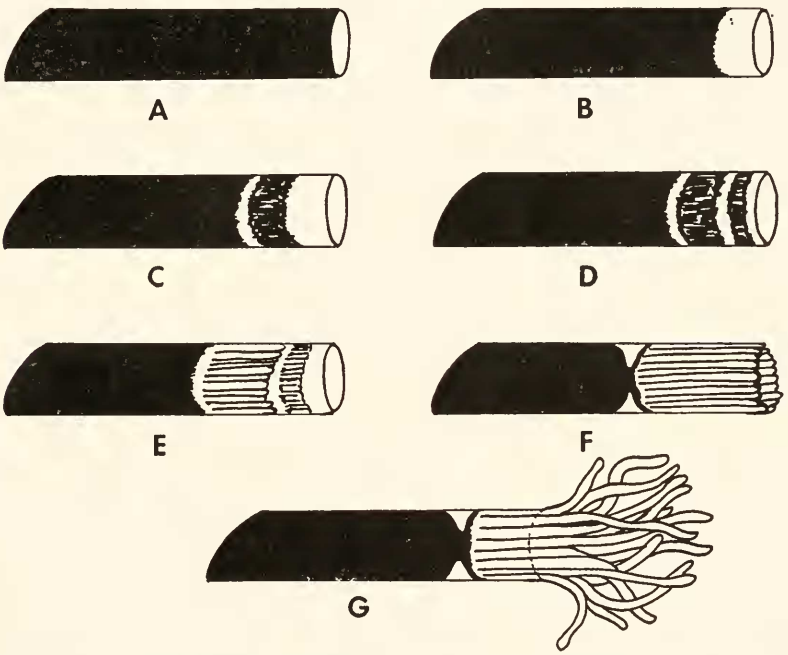


Figure 1. Stages in *Tubularia* regeneration. A, inactive; B, activated; C, one-band; D, two-band; E, two-band striated; F, constricted; G, emerging.

Stems were individually cut from the colony. The distal end was severed 5 mm. from the hydranth perpendicular to the vertical axis of the stem; the proximal cut was made obliquely to the vertical axis of the stem 6 mm. from the distal end. The stems were placed in glass fingerbowls of filtered pasteurized sea water containing chloromycetin (100 mg./l.) to control bacterial growth and were allowed to develop under a flow (300 ml./min.) of filtered sea water. Cheesecloth covers prevented the stems from flowing out of the fingerbowls.

The stems were allowed to regenerate approximately 15 hours. Stems that were in the two-band striated stage (Fig. 1E) were individually selected. Thirty to 35 future hydranth regions were cut from these stems. These primordia were homogenized in 1 ml. of pasteurized filtered sea water.

The homogenate was centrifuged at 5500 *g* for 5 minutes. A portion of the supernatant was pipetted into a "well" ($8 \times 2 \times 4$ mm.) which had been cut in

electrophoretic starch. In addition to primordial homogenate, 0.05 ml. of each of the following four other solutions was electrophoresed: (1) 0.5 ml. of primordial supernatant + 0.08% trypsin in sea water, (2) 0.5 ml. of sea water + 0.08% trypsin in sea water, (3) 0.5 ml. of sea water + 0.30% trypsin in sea water, (4) sea water.

The starch was made with borate buffer (pH 8.6) which had been diluted 10 times with distilled water. Platinum electrodes transmitted the current from a Vokam Power Supply (type 2541) through the undiluted buffer in the electrode wells to filter paper wicks in contact with the starch. The gel was covered with a thin coating of paraffin oil to prevent drying and cracking of the blocks. Ice cubes were used to maintain the temperature at 15–16° C. Electrophoresis was carried out for three hours at 150 volts with a milliamperage of 5. The remaining super-

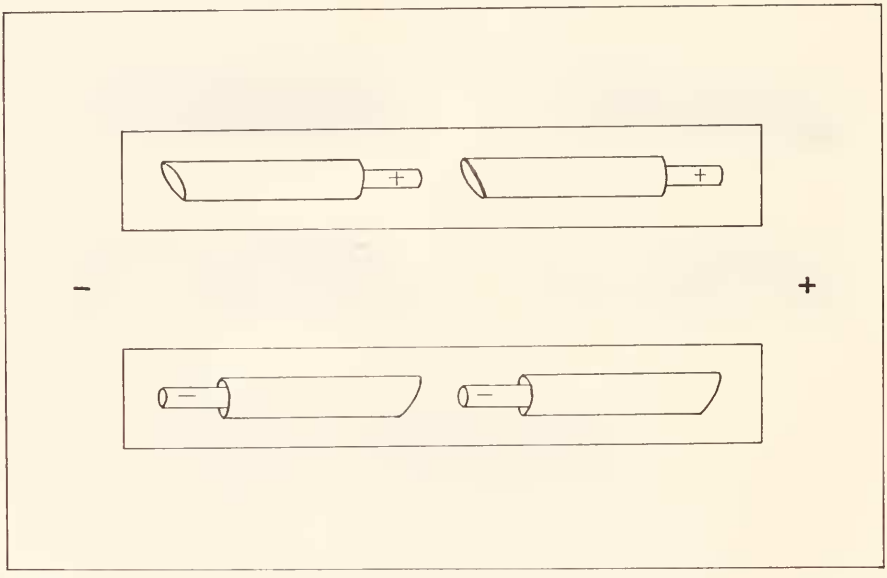


FIGURE 2. *Tubularia* stems containing starch cores with charged material oriented in electric field.

natant was pipetted from the well when electrophoresis was terminated. Duplicate blocks were simultaneously electrophoresed. The duplicates were subsequently stained with amido black and then destained to estimate the mobility of proteins through the gel. These blocks indicated that an area 5 mm. from the edge of the well might contain a relatively large amount of material that had been moved through the starch gel.

Glass coring tubes whose bores were 0.3–0.4 mm. were used to make plugs from the starch 5 mm. on both sides of the well. The starch gel core was forced out of the tube by applying heat to the larger opposite closed end of the coring tube. Starch plugs were made from the previously described electrophoresed blocks. They were inserted into the distal ends of *Tubularia* stems individually cut from a single colony. The stems selected had all reached the one-band stage in regeneration (Fig. 1C). The plugs were placed with approximately one-third of their total length within the coelenteron. Any stem which had been physically damaged

to the slightest extent during plugging was not used for experimental purposes.

The stems were placed in V-shaped grooves cut in a tray of 2% agar gel made with filtered pasteurized sea water containing chloromycetin (100 mg./l.). They were oriented so that any charged material in the plugs would be drawn into the animal when a current was applied (Fig. 2). (Pilot experiments had revealed that the plugged stems had to be placed in an electric current for the primordial extract gel to inhibit the rate of regeneration.)

A series of experiments was conducted to determine the current to which *Tubularia* could be subjected for 8 hours and still undergo typical regeneration. A current of 1 m.a./5.7 mm.$^2$ caused slowing of the rate of regeneration and some reversible damage to the stems. A current of 1 m.a./6.0 mm.$^2$ slightly retarded the rate of reconstitution. The current used in these experiments (1 m.a./68 mm.$^2$)

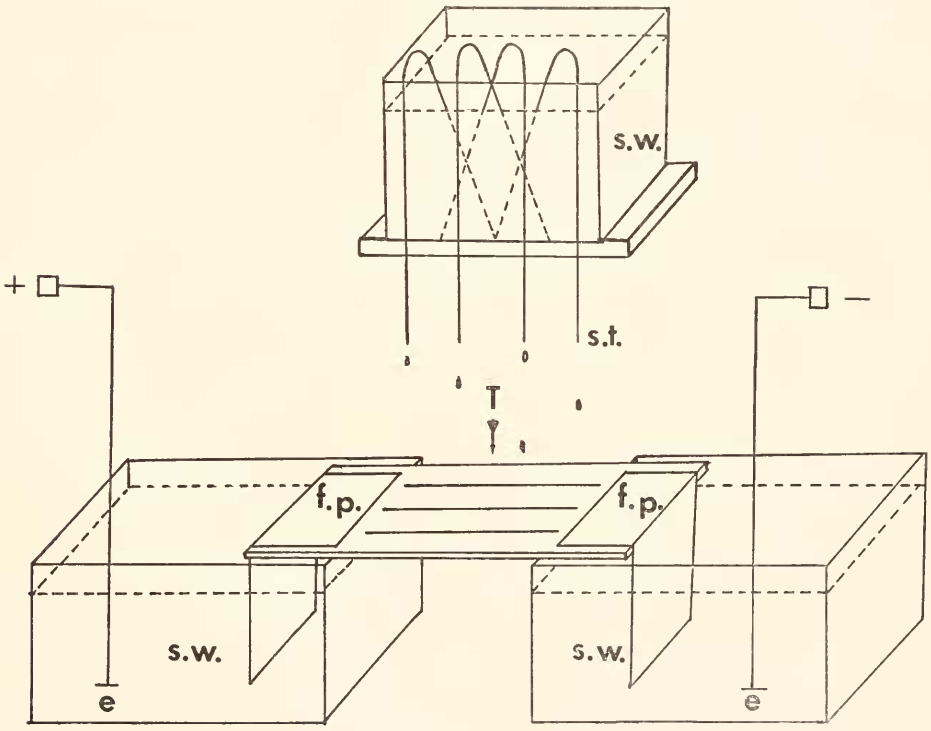


FIGURE 3. *Tubularia* in agar tray exposed to an electric field (6 volts; 10 milliamperes) for 8 hours. *e*, platinum electrode; *s.w.*, filtered pasteurized sea water containing chloromycetin; *f.p.*, filter paper wicks; T, *Tubularia* stems in grooves cut in tray of agar; *s.t.*, siphon tube.

did not visibly damage the stems and allowed typical regeneration. There was no evidence of the reversal of polarity which has been reported when higher currents were applied over longer periods (Barth, 1934; Levin, 1961).

A plastic tray (173 × 85 × 8 mm.) containing plugged *Tubularia* and a group of unplugged stems was placed in an electric current of 10 milliamperes and a voltage of 6 for eight hours (Fig. 3). The temperature was maintained at 15–16° C. during the entire eight hours. The stems were under a constant flow (100 ml./hr.) of filtered pasteurized sea water containing chloromycetin (100 mg./l.) This

TABLE I

*Experiment 1**

*Number of Tubularia in each regeneration stage eight hours after plugging*

| Type of electrophoresed material contained in the plugs | Inactive | Activated | 1-Band | 2-Band | 2-Band striated | Constricted | Emerging |
|---|---|---|---|---|---|---|---|
| (+) Primordia | 5 | 1 | 1 | 0 | 1 | 2 | 0 |
| (−) Primordia | 5 | 1 | 1 | 1 | 0 | 0 | 0 |
| (+) Primordia & 0.08% trypsin | 1 | 0 | 0 | 0 | 1 | 6 | 0 |
| (−) Primordia & 0.08% trypsin | 1 | 0 | 0 | 0 | 2 | 5 | 0 |
| (+) Sea water & 0.08% trypsin | 1 | 1 | 0 | 1 | 0 | 5 | 0 |
| (−) Sea water & 0.08% trypsin | 2 | 1 | 0 | 1 | 1 | 3 | 0 |
| (+) Sea water & 0.30% trypsin | 1 | 2 | 0 | 0 | 1 | 3 | 1 |
| (−) Sea water & 0.30% trypsin | 3 | 0 | 0 | 1 | 0 | 4 | 0 |
| (+) Sea water | 2 | 0 | 0 | 1 | 2 | 2 | 1 |
| (−) Sea water | 1 | 0 | 0 | 0 | 0 | 5 | 1 |
| Unplugged controls | 1 | 0 | 0 | 1 | 0 | 6 | 0 |

* This experiment was repeated three more times with similar results.

flow was delivered by multiple overhead capillary siphon tubes. The electrode wells were filled with sea water.

At the end of eight hours the stems were removed from the current and classified into one of seven regenerative stages (Fig. 1): inactive (newly cut), activated, one-band, two-band, two-band striated, constricted, and emerging. Davidson and Berrill (1948) and Steinberg (1954) described detailed characteristics of the stages of *Tubularia* regeneration. Over 325 stems were studied in these experiments. The stems were placed in stender dishes at 15° C. for further observations.

## RESULTS

The regeneration stages reached by stems eight hours after plugging in the first experiment are indicated in Table I. These experiments were repeated four

TABLE II

*Percentage of stems at two-band or a more advanced stage of regeneration eight hours after plugging.*
*(Means of four experiments)*

| Type of electrophoresed material contained in the plugs | Number of stems | Mean percentage |
|---|---|---|
| (+) Primordia | 33 | 49 |
| (−) Primordia | 36 | 32 |
| (+) Primordia & 0.08% trypsin | 31 | 93 |
| (−) Primordia & 0.08% trypsin | 32 | 84 |
| (+) Sea water & 0.08% trypsin | 32 | 75 |
| (−) Sea water & 0.08% trypsin | 32 | 62 |
| (+) Sea water & 0.30% trypsin | 24 | 62 |
| (−) Sea water & 0.30% trypsin | 22 | 68 |
| (+) Sea water | 28 | 71 |
| (−) Sea water | 28 | 85 |
| Unplugged controls | 31 | 83 |
| Total | 329 | |

times with similar results. The time involved in making the plugs and inserting them in the stems limited the number of stems involved in the experiment. Because this phase of the investigation was conducted over a five-week period and the regenerative rates of controls varied from colony to colony, it was not valid to designate stage-by-stage summation of all four experiments in a single chart. However, Table II indicates the mean percentage of stems in all four experiments that had reconstituted to a more advanced stage than the one-band stage they exhibited when the cores were inserted.

*Tubularia* plugged with gel from electrophoresed sea water regenerated at a rate comparable to those which were not plugged with any type of gel. The animals that received gel from electrophoresed homogenates of primordia regenerated at approximately half the rate of the controls. When the primordial material was combined with 0.08% trypsin, the stems developed at the same rate as the controls. The other two groups plugged with sea water plus trypsin starch gel indicated that trypsin did not increase the rate of regeneration; in fact, it somewhat retarded the rate of regeneration.

From these results it was concluded that a substance (or substances) from the supernatant of primordial tissue homogenate and separated by starch gel electrophoresis is capable of inhibiting the regeneration rate of *Tubularia*. The effect of this substance may be removed by the addition of a small amount of trypsin, thus indicating a polypeptide or protein nature for this inhibiting factor.

## Discussion

In these experiments the factors which are known to influence the regenerative process have been controlled. Any slight temperature variations (Moog, 1941) were simultaneously experienced by all stems in each experiment, and thus did not cause the differences observed in rate of reconstitution. A constant uniform flow of sea water was maintained in an effort to provide constant oxygen tension (Miller, 1937, 1939; Barth, 1938, 1940; Rose and Rose, 1941) and hydrogen ion concentration (Miller, 1939; Goldin, 1942). Chloromycetin was added to the environment to control bacterial growth (Tweedell, 1958b). The low currents (Barth, 1934; Levin, 1961) used did not cause a reversal of polarity, nor did they inhibit the rate of regeneration.

The substance (or substances), which is moveable in an electric field, can act as an inhibitor of regeneration. This factor does not totally inhibit reconstitution in the concentration employed, although it often caused the stems to regress temporarily to a less differentiated stage (Table I). When these stems were removed from the current they proceeded to differentiate in a typical manner. Subsequent observations revealed that the same percentage of the inhibited stems eventually emerged in the inhibited groups and in the control groups.

Plugs containing homogenate from two-band striated stems were tested for their effect on the earlier one-band stage. This method was used because preliminary experiments indicated that homogenates from primordia of early regeneration stages did not inhibit development in later stages. Steinberg (1954) and Tweedell (1958a) found that the inhibitor acts only during early stages of regeneration.

Some stems plugged with gel containing electrophoresed primordial supernatant continued to regenerate at a rate similar to that of the controls (Tables I and II). There are three principal factors that may have contributed to this observation: (1) The concentration of inhibiting substance in some plugs may not have been sufficient to cause an observable effect. (2) *Tubularia* stems exhibit individual variation in their rate of reconstitution; therefore, some stems that would have developed rapidly could have been inhibited, but still have regenerated at the same rate as slower regenerating control stems. (3) There seems to be a critical period for using inhibitor substance obtained from one development stage on a less advanced stage. Some stems may have progressed beyond the period when the inhibitor substance could exert its optimal action.

Inhibitory material from primordia was found on both sides of the well. This could be explained by the presence of two similar, but differently charged inhibitors. It is well known that proteins exist in nature only as part of more complex systems. Indeed, even purified proteins may be formed by the union of several simpler components. Another possibility is that the inhibitor is a substance that is altered in some way so that it is fractionated into two active oppositely charged components.

A group of pilot experiments which were performed at pH 4.1 using acetate buffer indicated that the substance (or substances) was positively charged at this pH since no inhibitory material could be detected migrating toward the positive pole. More experiments at different pH's using techniques of finer separation will be needed to elucidate the chemical properties of the inhibitor.

Rose (1963) discussed the polarized control of differentiation during regeneration. He emphasized the hypothesis that (p. 490) "bioelectric fields determine the direction of flow of specific inhibitors, thus changing a totipotent system into a differentiated one."

The findings in these experiments lend support to Rose's hypothesis: *viz.* they confirm that there is an inhibitory agent (or agents) of a polypeptide or protein nature that can be moved in an electric field.

## Summary

1. Supernatant of homogenate obtained from regenerating *Tubularia* stems was electrophoresed through starch gel.

2. Starch cores from the gel were partially inserted into the coelenterons of stems in an earlier stage of reconstitution.

3. These stems were then oriented in an electric current so that any charged material in the cores would migrate toward the stems.

4. The electrophoresed supernatant from primordial homogenate retarded the rate of reconstitution.

5. The addition of a small amount of trypsin prior to electrophoresis removed the regeneration-inhibiting effect of the supernatant, thus indicating a polypeptide or protein nature for the inhibiting substance(s).

## LITERATURE CITED

Barth, L. G., 1934. The effect of constant electric current on regeneration of certain hydroids. *Physiol. Zoöl.*, **7**: 340–364.

Barth, L. G., 1938. Oxygen as a controlling factor in the regeneration of *Tubularia*. *Physiol. Zoöl.*, **11**: 179–186.

Barth, L. G., 1940. The role of oxygen in regeneration in *Tubularia*. *Biol. Bull.*, **79**: 360.

Child, C. M., 1941. Patterns and Problems of Development. Chicago: University of Chicago Press.

Davidson, M. E., and N. J. Berrill, 1948. Regeneration of primordia and developing hydranths of *Tubularia*. *J. Exp. Zool.*, **132**: 555–574.

Fulton, C., 1959. Re-examination of an inhibitor of regeneration in *Tubularia*. *Biol. Bull.*, **116**: 232–238.

Goldin, A., 1942. A quantitative study of the interrelationship of oxygen and hydrogen ion concentration in influencing *Tubularia* regeneration. *Biol. Bull.*, **82**: 340–346.

Levin, S., 1961. Anodal inhibition of the regeneration of *Tubularia crocea* within an electric current. *Biol. Bull.*, **121**: 395.

Miller, J. A., Jr., 1937. Some effects of oxygen on polarity in *Tubularia*. *Biol. Bull.*, **73**: 369.

Miller, J. A., Jr., 1939. Experiments on polarity determination in *Tubularia* regenerates. *Anat. Rec.*, **94**: 17.

Moog, F., 1941. The influence of temperature on reconstitution in *Tubularia*. *Biol. Bull.*, **81**: 300–301.

Powers, J. A., 1961. Effects of distal and proximal hydranth extracts and stem extracts from adult *Tubularia* on the regeneration of isolated proximal ridge regions. *Biol. Bull.*, **121**: 404.

Rose, S. M., 1955. Specific inhibition during differentiation. *Ann. N. Y. Acad. Science*, **60**: 1136–1159.

Rose, S. M., 1957. Polarized inhibitory effects during regeneration in *Tubularia*. *J. Morph.*, **100**: 187–205.

Rose, S. M., 1961. Polarized control of regeneration in *Tubularia* by charged particles. *Biol. Bull.*, **121**: 405.

Rose, S. M., 1963. Polarized control of regional structure in *Tubularia*. *Dev. Biol.*, **7**: 488–501.

Rose, S. M., and F. C. Rose, 1941. The role of a cut surface in *Tubularia* regeneration. *Physiol. Zoöl.*, **14**: 328–343.

Steinberg, M. S., 1954. Studies on the mechanism of physiological dominance in *Tubularia*. *J. Exp. Zool.*, **127**: 1–26.

Tweedell, K. S., 1958a. Inhibitory control of regeneration in *Tubularia*. *Biol. Bull.*, **114**: 225–269.

Tweedell, K. S., 1958b. A bacteria-free inhibitor of regeneration in *Tubularia*. *Biol. Bull.*, **115**: 369.

Tweedell, K. S., 1962. Inhibition of regeneration in *Tubularia* by tissue extract injection *Biol. Bull.*, **123**: 513–514.